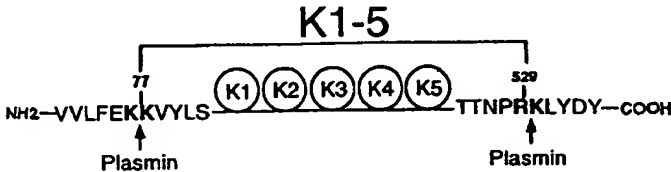
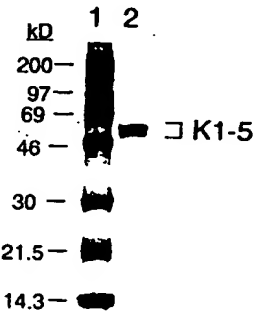


WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

<p>(51) International Patent Classification <sup>6</sup> :  <b>C07K 14/47, A61K 38/17</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 99/00420</b></p> <p>(43) International Publication Date: <b>7 January 1999 (07.01.99)</b></p>									
<p>(21) International Application Number: <b>PCT/SE98/01262</b></p> <p>(22) International Filing Date: <b>26 June 1998 (26.06.98)</b></p> <p>(30) Priority Data:</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">60/050,822</td> <td style="width: 30%;">26 June 1997 (26.06.97)</td> <td style="width: 40%;">US</td> </tr> <tr> <td>60/055,971</td> <td>18 August 1997 (18.08.97)</td> <td>US</td> </tr> <tr> <td>9703057-1</td> <td>25 August 1997 (25.08.97)</td> <td>SE</td> </tr> </table> <p>(71) Applicant (for all designated States except US): <b>KAROLIN-SKA INNOVATIONS AB [SE/SE]; S-171 77 Stockholm (SE).</b></p> <p>(72) Inventor; and  (75) Inventor/Applicant (for US only): <b>YIHAI, Cao [SE/SE]; Åsögatan 184 IV, S-116 32 Stockholm (SE).</b></p> <p>(74) Agents: <b>BERG, Sven, Anders et al.; Albihns Patentbyrå Stockholm AB, P.O. Box 3137, S-103 62 Stockholm (SE).</b></p>			60/050,822	26 June 1997 (26.06.97)	US	60/055,971	18 August 1997 (18.08.97)	US	9703057-1	25 August 1997 (25.08.97)	SE
60/050,822	26 June 1997 (26.06.97)	US									
60/055,971	18 August 1997 (18.08.97)	US									
9703057-1	25 August 1997 (25.08.97)	SE									
<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b>  <i>With international search report.</i></p>											
<p>(54) Title: <b>KRINGLE DOMAINS 1-5 OF PLASMINOGEN, CAPABLE OF MODULATING ANGIOGENESIS IN VIVO</b></p> <p>(57) Abstract</p> <p>The present invention relates to a protein capable of regulating the endothelial cell proliferation as well as various uses thereof. The protein according to the invention has a molecular weight of approximately 50-60 kDa, preferably about 55 kDa, and has an amino acid sequence, which is substantially similar to that of a plasminogen fragment comprised of Lys 78-Arg 530 of a plasminogen molecule.</p>											
<p><b>A</b></p> 											
<p><b>B</b></p> 											

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## KRINGLE DOMAINS 1-5 OF PLASMINOGEN, CAPABLE OF MODULATING ANGIOGENESIS IN VIVO

5

Field of the Invention

The present invention relates to novel endothelial cell proliferation inhibitors. The inhibitors are extraordinarily potent in inhibiting angiogenesis related diseases and modulating angiogenic processes. In addition, the present  
10 invention relates to methods of treating a human or animal having an angiogenic disease by administering an inhibitor according to the invention thereto.

Background

15 As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ, and involves endothelial cell proliferation. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. The  
20 term "endothelium" means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels and blood vessels.

Both controlled and uncontrolled angiogenesis are thought to proceed in a  
25 similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants  
30 induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, whe-

re the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

5 Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells and supports the pathological damage seen in these conditions. The diverse pathological disease states in which unregulated angiogenesis is present have been grouped together as angiogenic dependent or angiogenesis associated diseases.

10

The hypothesis that tumor growth is angiogenesis-dependent was first proposed in 1971. (Folkman J., Tumor angiogenesis: Therapeutic implications. *N. Engl. Jour. Med.* 285:1182-1186, 1971). In its simplest terms it states: "Once tumor "take" has occurred, every increase in tumor cell population  
15 must be preceded by an increase in new capillaries converging on the tumor. Tumor "take" is currently understood to indicate a prevascular phase of tumor growth in which a population of tumor cells occupying a few cubic millimeters volume, and not exceeding a few million cells, can survive on existing host microvessels. Expansion of tumor volume beyond this phase  
20 requires the induction of new capillary blood vessels. For example, pulmonary micrometastases in the early prevascular phase in mice would be undetectable except by high power microscopy on histological sections.

Examples of the indirect evidence which support this concept include:

25 (1) The growth rate of tumors implanted in subcutaneous transparent chambers in mice is slow and linear before neovascularization, and rapid and nearly exponential after neovascularization. (Algire GH, et al. Vascular reactions of normal and malignant tumors *in vivo*. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants.; *J Natl. Cancer*  
30 Inst.6:73-85,1945).

- (2) Tumors grown in isolated perfused organs where blood vessels do not proliferate are limited to 1-2 mm<sup>3</sup> but expand rapidly to > 1000 times this volume when they are transplanted to mice and become neovascularized. (Folkman J, et al., Tumor behavior in isolated perfused organs: *In vitro* growth and metastasis of biopsy material in rabbit thyroid and canine intestinal segments. *Annals of Surgery* 164:491-502, 1966).
- (3) Tumor growth in the avascular cornea proceeds slowly and at a linear rate, but switches to exponential growth after neovascularization. (Gimbrone, M.A., Jr. et al., Tumor growth and neovascularization: An experimental model using the rabbit cornea. *J. Natl. Cancer Institute* 52:41-427, 1974).
- (4) Tumors suspended in the aqueous fluid of the anterior chamber of the rabbit eye, remain viable, avascular and limited in size to < 1 mm<sup>3</sup>. Once they are implanted on the iris vascular bed, they become neovascularized and grow rapidly, reaching 16,000 times their original volume within 2 weeks. (Gimbrone MA Jr., et al., Tumor dormancy *in vivo* by prevention of neovascularization, *J. Exp. Med.* 136:261-276).
- (5) When tumors are implanted on the chick embryo chorioallantoic membrane, they grow slowly during an avascular phase of >72 hours, but do not exceed a mean diameter of 0.93 + 0.29 mm. Rapid tumor expansion occurs within 24 hours after the onset of neovascularization, and by day 7 these vascularized tumors reach a mean diameter of 8.0 + 2.5 mm. (Knighton D., Avascular and vascular phases of tumor growth in the chick embryo. *British J. Cancer*, 35:347-356, 1977).
- (6) Vascular casts of metastases in the rabbit liver reveal heterogeneity in size of the metastases, but show a relatively uniform cut-off point for the size at which vascularization is present. Tumors are generally avascular up to 1 mm in diameter, but are neovascularized beyond that diameter. (Lien W., et al., The blood supply of experimental liver metastases. II. A microcircu-

latory study of normal and tumor vessels of the liver with the use of perfused silicone rubber. *Surgery* 68:334-340,1970).

- (7) In transgenic mice which develop carcinomas in the beta cells of the pancreatic islets, pre-vascular hyperplastic islets are limited in size to < 1 mm<sup>3</sup>. At 6- 7 weeks of age, 4- 10% of the islets become neovascularized, and from these islets arise large vascularized tumors of more than 1000 times the volume of the pre-vascular islets. (Folkman J, et al., Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* 339:58-61,1989).
- 5 (8) A specific antibody against VEGF (vascular endothelial growth factor) reduces microvessel density and causes "significant or dramatic" inhibition of growth of three human tumors which rely on VEGF as their sole mediator of angiogenesis (in nude mice). The antibody does not inhibit growth of the tumor cells *in vitro*. (Kim K J, et al., Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. *Nature* 362:841-844,1993).
- 10 (9) Anti-bFGF monoclonal antibody causes 70% inhibition of growth of a mouse tumor which is dependent upon secretion of bFGF as its only mediator of angiogenesis. The antibody does not inhibit growth of the tumor cells *in vitro*. (Hori A, et al., Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. *Cancer Research*, 51:6180-6184, 1991).
- 15 (10) Intraperitoneal injection of bFGF enhances growth of a primary tumor and its metastases by stimulating growth of capillary endothelial cells in the tumor. The tumor cells themselves lack receptors for bFGF, and bFGF is not a mitogen for the tumor cells *in vitro*. (Gross JL, et al., Modulation of solid tumor growth *in vivo* by bFGF. *Proc. Amer. Assoc. Canc. Res.* 31:79,1990).
- 20 (11) A specific angiogenesis inhibitor (AGM-1470) inhibits tumor growth and metastases *in vivo*, but is much less active in inhibiting tumor cell proliferation *in vitro*. It inhibits vascular endothelial cell proliferation half-
- 25
- 30

maximally at 4 logs lower concentration than it inhibits tumor cell proliferation. (Ingber D, et al., Anaioinhibins: Synthetic analogues of fumagillin which inhibit angiogenesis and suppress tumor growth. *Nature*, 48:555-557, 1990). There is also indirect clinical evidence that tumor growth is angiogenesis dependent.

(12) Human retinoblastomas that are metastatic to the vitreous develop into avascular spheroids which are restricted to less than 1 mm<sup>3</sup> despite the fact that they are viable and incorporate <sup>3</sup>H-thymidine (when removed from an enucleated eye and analyzed *in vitro*).

(13) Carcinoma of the ovary metastasizes to the peritoneal membrane as tiny avascular white seeds (1-3 mm<sup>3</sup>). These implants rarely grow larger until one or more of them becomes neovascularized.

(14) Intensity of neovascularization in breast cancer (Weidner N, et al., Tumor angiogenesis correlates with metastasis in invasive breast carcinoma. *N. Engl. J. Med.* 324:1-8, 1991, and Weidner N, et al., Tumor angioaenesis: A new significant and independent prognostic indicator in early-stage breast carcinoma, *J Natl. Cancer Inst.* 84:1875-1887, 1992) and in prostate cancer (Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *American Journal of Pathology*, 143(2):401-409, 1993) correlates highly with risk of future metastasis.

(10) Metastasis from human cutaneous melanoma is rare prior to neovascularization. The onset of neovascularization leads to increased thickness of the lesion and an increasing risk of metastasis. (Srivastava A, et al., The prognostic significance of tumor vascularity in intermediate thickness (0.76-4.0 mm thick) skin melanoma. *Amer. J. Pathol.* 133:419-423, 1988)

(16) In bladder cancer, the urinary level of an angiogenic peptide, bFGF, is a more sensitive indicator of status and extent of disease than is cytology. (Nguyen M, et al., Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in urine of bladder cancer patients. *J. Natl. Cancer Inst.* 85:241-242, 1993).

Thus, it is clear that angiogenesis plays a major role in the metastasis of a cancer. If this angiogenic activity could be repressed or eliminated, or otherwise controlled and modulated, then the tumor, although present, would not grow. In the disease state, prevention of angiogenesis could avert the damage caused by the invasion of the new microvascular system. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

Accordingly, within this field, there is a strong need for compositions and methods by which endothelial cell proliferation, such as the unwanted growth of blood vessels, especially into tumors, may be inhibited. There is also a need for methods for detecting, measuring and localizing such compositions. Such compositions should be able to overcome the activity of endogenous growth factors in premetastatic tumors and prevent the formation of the capillaries in the tumors, thereby inhibiting growth of the tumors. In addition, the compositions, fragments of such compositions and antibodies specific to said compositions should be able to modulate the formation of capillaries in other angiogenic processes, such as wound healing and reproduction. Naturally, compositions and methods for inhibiting angiogenesis should preferably be non-toxic and produce few side effects. Also needed is a method for detecting, measuring and localizing the binding sites for the composition as well as sites of biosynthesis of the composition. The compositions and fragments of the compositions should be capable of being conjugated to other molecules for both radioactive and non-radioactive labeling purposes.

#### Prior art

It has been suggested to use the first four kringle regions, nos. 1-4, of plasminogen, also denoted angiostatin, as a composition for the above defined purposes. However, the inhibitory effect of angiostatin on endothelial cell



proliferation is too low to be satisfactory. Used as a medicament for human patients, angiostatin would have to be administered in kilograms to be effective, which of course would put severe limitations to the use thereof due to practical reasons. Another drawback lies in the amounts which would be necessary to produce due to said low effect, making such a product quite costly. Thus, in spite of the discovery of angiostatin, there is still a strong need for a composition which fulfills the objects defined above.

#### Summary of the Invention

The object of the present invention is to fulfill above defined need. This is accomplished by the present invention, which relates to a protein capable of modulating or regulating, e.g. inhibiting, the endothelial cell proliferation in *in vitro* and angiogenesis in *in vivo* assays. The invention also relates to any nucleic acid encoding such a protein. The various further aspects of the invention will be described in detail below with reference to the drawings.

#### Brief Description of the Drawings

Figure 1 shows a proteolytic human kringle 1-5 (K1-5) fragment, schematically and purified on a gel.

Figure 2 A-B show the anti-endothelial cell proliferation activity of K1-5, while 2 C-D show the inhibition of capillary endothelial cell proliferation. Figure 3 shows the morphology of K 1-5 treated endothelial cells. Figure 4 shows the synergistic suppression of endothelial cell growth by angiostatin and K5.

Figure 5 shows the anti-angiogenic effect of K 1-5 according to the invention on the chick embryo chorioallantoic membrane (CAM).

Figure 6 shows suppression of primary tumor growth by K 1-5 according to the invention, comparing mice treated with K 1-5 with saline treated mice.

Figure 7 shows detection of microvessel density by immunohistochemical staining of tumor tissue sections with anti-vWF antibodies.

Detailed description of the invention

In a first aspect, the present invention relates to a protein as defined by claim 1. Generally, the protein according to the invention is comprised of at least about 50% of the amino acid sequence which the human kringle 1-5 sequence as disclosed in SEQ ID NO. 1 and the mouse kringle 1-5 as disclosed in SEQ ID NO. 2 has in common. In a more specific embodiment, the protein according to the invention comprises about 60-70% of the amino acid sequence which SEQ ID NO 1 and 2 has in common and more specifically essentially all of that sequence. In one particular embodiment, the protein according to the invention is comprised of essential parts the human amino acid sequence and in an alternative embodiment, the protein according to the invention is comprised of essentially all of the mouse amino acid sequence. It is to be understood that in all embodiments of the protein according to the invention, analogues and functional fragments thereof are also encompassed.

In one embodiment, the protein according to the invention has a molecular weight of between about 50 to about 65 kilodaltons, as determined by non-reducing polyacrylamide gel electrophoresis. The molecular weight depends *inter alia* on whether the molecule has been glycosylated or not, which in turn depends on how it has been produced. The molecule according to the invention exhibits an amino acid sequence which is substantially similar to that of a plasminogen fragment comprised of Lys 78-Arg 530 of a plasminogen molecule. In the literature, the identity of the amino acids with which the plasminogen starts and ends have been debated. However, in the present context, the most important feature of the molecule according to the invention is that it possesses the advantageous angiogenic properties described herein. Thus, the sequence which the present molecule resembles may start with Lys 77 or Lys 78 and, correspondingly, it may end with Arg 529 or Arg 530. A preferred embodiment of the invention is a molecule, which mo-

re specifically has a molecular weight of about 50 to about 60 kilodalton, preferably about 53 to about 57 kilodalton and most preferred about 55 kilodalton. The plasminogen fragment according to the broad definition given above, i.e. comprised of the amino acid sequence Lys78-Arg 530 of plasminogen, is also denoted K1-5, as it comprises the first five kringle domains of plasminogen. Even though the fifth domain indeed has been known to exist before, the general opinion of the experts within this field has been that kringle no. 5 has not contributed to, or shared, the angiogenic properties of angiostatin. Thus, a fragment consisting of all the regions 1-5 of plasminogen has never been tested in total before as regards the inhibition of the growth of endothelial cells. The present inventor has now been able to disclose extremely promising results for such new fragments, or molecules exhibiting sequences substantially similar to such fragments, which compared to the prior angiostatin compositions quite surprisingly are superior in the present context. In addition to the advantages mentioned above, the molecule according to the invention is also more preferred than the prior angiostatin compounds, since the present molecule is larger. Consequently, the present molecule is more stable and therefore more favourable for use as a medicament and in medicinal compositions.

20

The present invention relates to a molecule as defined above, wherein the plasminogen fragment is similar to a fragment selected from the group consisting of human plasminogen, murine plasminogen, bovine plasminogen, Rhesus plasminogen and porcine plasminogen. The molecule, or protein, according to the present is capable of inhibiting endothelial cell proliferation in *in vitro* assays.

25

In one embodiment of the protein according to the invention, the protein comprises essentially all of the sequence of SEQ ID NO. 1 or any functional fragment or analogue thereof. In an alternative embodiment, the protein comprises essentially all of the sequence of SEQ ID NO. 2 or any functional

30

fragment or analogue thereof. Said function relates to the advantageous endothelial cell proliferation regulating property that according to the invention has been associated with K1-5 of plasminogen.

- 5 Another object of the present invention is the use of a molecule according to the invention, such as K1-5, as a medicament. In addition, the invention also relates to the use of a molecule as defined above for the manufacture of a medicament for modulating, *e.g.* inhibiting, endothelial cell proliferation, for example for treating angiogenesis associated conditions or diseases,  
10 such as tumor growth, *e.g.* cancer, diabetes *etc.*

- Accordingly, the invention also relates to a pharmaceutical or veterinary composition, which comprises a molecule according to the invention as well as one or more pharmaceutically acceptable carriers and/or excipients.
- 15 The composition may be administered in a variety of unit dosage forms depending upon the method of administration, *e.g.* parenteral, topical, oral or local administration, for prophylactic and/or therapeutic treatment. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and lozenges. A variety of carriers may be used, such  
20 as aqueous carriers, *e.g.* buffered saline *etc.* These solutions are free of undesirable matter. The compositions may also include pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents and the like, *e.g.* sodium acetate, sodium chloride, potassium chloride, calcium chloride *etc.* For parenterally administrable compositions, see *e.g.*  
25 *Remington's Pharmaceutical Science*, 15<sup>th</sup> ed., Mack Publishing Company, Easton, Pennsylvania (1980). A composition, or preparation, according to the present invention may be administered in a much lower dosage and the use thereof is thus superior to that of known angiostatin compositions.
- 30 Consequently, the use of a pharmaceutical preparation comprising K1-5, when compared to use of angiostatin, is easier to administer due to the

smaller amount needed, which smaller dose also results in a cheaper medicament. In one embodiment of the invention, the composition comprises a molecule or protein, which is capable of inhibiting cancer metastases. The half maximal concentration (EC50) of the molecule according to the present invention for the inhibition of endothelial cell proliferation is about 50 pM, to be compared with the EC50 value for angiostatin, which is 130 nM. Thus, there is a 500-1000 fold difference between the effects of the compositions according to the invention and the effect of angiostatin, which is remarkable and highly surprising. Indeed, when the inhibitory effects of the first four kringle domains of plasminogen, angiostatin, were evaluated individually and combined, it was found that the most potent endothelial cell proliferation inhibition resulted when kringle region 4 was removed from angiostatin. (See The Journal of Biological Chemistry, 1996, vol 271, No 46: "Kringle Domains of Human Angiostatin", Cao *et al.*) . Thus, the general opinion within this area has been that a more potent fragment would result if kringle no 4 was removed from the protein. However, surprisingly, the present inventors have now shown that not only did the previously believed inactive kringle 5 of plasminogen show effect, but, also, that the new molecule also include the kringle 4, which has been known to lower the effect of the other angiostatin fragments. As mentioned above, the molecule according to the invention is also more stable in the human or animal body than the prior art compounds, probably due to a higher resistance against proteases and other enzymes present. In other words, it exhibits a longer half-time, which is advantageous as it enables a less frequent administration as well as lower amounts. Thus, a K1-5 preparation according to the present invention is more convenient for the patient treated therewith and also less costly than the angiostatin or K5 compounds of the prior art.

Another object of the present invention is a nucleic acid, such as a DNA or RNA, encoding any molecule according to the invention. A cDNA sequence which is complementary to such a sequence is also encompassed. Thus,

further object of the invention is any nucleic acid which under stringent conditions hybridizes specifically to one of the above defined nucleic acids. In the present context, the term "hybridising specifically to" refers to the binding, duplexing or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture of DNA or RNA. In the present context, the term "stringent conditions" refers to conditions, under which a probe will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. The one skilled in this field will easily choose the suitable conditions in the present context. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T<sub>m</sub>) for the specific sequence at a defined ionic strength and pH. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, such as about 0.01-1.0 M, at a pH of about 7.0-8.3 and the temperature is between about 30°C and 60°C, depending on the length of the nucleotide. Stringent conditions may also be achieved by the addition of destabilizing agents, such as formamide. Such a nucleotide according to the invention may be of any length in accordance with the above defined.

The nucleic acids according to the invention are cloned or amplified by in vitro methods, such as polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), etc. A wide variety of cloning and in vitro amplification methods are well known to persons of skill, see e.g. Berger and Kimmel, *Guide to Molecular Cloning Techniques*, Methods in Enzymology 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook et al., (1989) *Molecular Cloning - A Laboratory Manual*, vol 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY; *Current Protocols in Molecular Biology*, F.M. Ausbel et al., eds., Current Protocols; Cashion et al., US patent no. 5 017 478; and Carr, EP patent no. 0 246 864.

In another aspect, the invention relates to a peptide, polypeptide or protein encoded by a nucleic acid according to the invention. General methods of preparation of such molecules is further disclosed below in the section regarding uses.

5

Another aspect of the present invention is an antibody raised against a peptide, polypeptide or protein according to the invention. Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (e.g. GST, keyhole limpet hemanocyanin etc.), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (see US patent no. 4 722 848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of the reactivity to the polypeptide of interest.

When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired (see. e.g. Coligan (1991) *Current Protocols in Immunology*, Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A laboratory manual*, Cold Spring Harbor Press, NY). In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans etc. Description of techniques for preparing such monoclonal antibodies are found in e.g. Stites et al., (eds.) *Basic and Clinical Immunology* (4<sup>th</sup> ed), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *supra*; Goding (1986), *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497.

30

An additional object is the use of a molecule as defined above in gene therapy as well as such gene therapy methods. The methods according to the invention can involve transfecting cells of a mammal with a vector expressing a polypeptide or antibody according to the invention. The transfection

tion can be *in vivo* or *ex vivo*. *Ex vivo* transfection is suitably followed by re-infusing the cells into the organism. Other methods involve administering to the mammal, e.g. a human, of a therapeutically effective dose of a composition comprising a polypeptide according to the invention and a pharmacological excipient and/or carrier.

For a review of gene therapy procedures, see Anderson, *Science* (1992) 256:808-813; Nabel and Felgner (1993) *TIBTECH* 11: 211-217; Mitani and Caskey (1993) *TIBTECH* 11: 162-166; Mulligan (1993) *Science* 926-932; Dillon (1993) *TIBTECH* 11: 167-175; Miller (1992) *Nature* 357: 455-460; Van Brunt (1988) *Biotechnology* 6(10): 1149-1154; Vigne (1995) *Restorative Neurology and Neuroscience* 8: 35-36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1) 31-44; Haddada *et al.* (1995) in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) Springer-Verlag, Heidelberg Germany; and Yu *et al.*, *Gene Therapy*(1994) 1:13-26.

One further object of the present invention is to provide a method of treating diseases and processes that are mediated by endothelial cell proliferation, especially angiogenesis. One such disease which may be treated is cancer. Accordingly, the invention relates to a method of treating a human or animal with an angiogenic disease comprising administering, to said human or animal, a molecule or protein having a molecular weight of approximately 50 - 65 kilodaltons as determined by non-reducing polyacrylamide gel electrophoresis and having an amino acid sequence substantially similar to that of a plasminogen fragment comprised of Lys 78-Arg 530 of a plasminogen molecule. In a preferred embodiment, the protein has a molecular weight of approximately 50 - 60 kilodaltons, preferably 53 -57 kilodalton and most preferred approximately 55 kilodalton.

The method according to the invention may use a molecule substantially similar to a plasminogen fragment, which is selected from the group consis-



ting of human plasminogen, murine plasminogen, bovine plasminogen, Rhesus plasminogen and porcine plasminogen.

Thus, the present invention encompasses protein molecules analogues of  
5 kringle 1-5 of plasminogen as well as methods of use thereof. The new part which has been added to the previously known angiostatin molecule is accordingly kringle no 5 of plasminogen, which is separately described in detail below. Previous tests have shown that kringle no 5 of plasminogen possesses a certain inhibitory effect alone (see US Patent Application No  
10 08/763 528, filed on December 12, 1996, by the present inventor, which application was secret at the time of the filing of the present application and which is hereby included via reference). However, the effect of K5 alone is hundreds of times weaker than the effect of the molecule according to the present invention. In addition, the endothelial cell proliferation inhibitory  
15 effect of the molecule according to the present invention is far superior than a mere addition of the effects of K1-4 and K-5.

The present invention also encompasses diagnostic and therapeutic methods for detecting the presence or absence of the inhibiting peptide in body  
20 fluids, and for administration of the peptide or antibodies that specifically bind the peptide to patients in need of therapeutically effective amounts of such compounds to regulate endothelial cell proliferation. Additionally, the inhibitory peptide may be used in conjunction with *in vitro* proliferating endothelial cell cultures to test for compounds that mitigate the inhibitory effects of the peptide - i.e. to screen for growth factors or other compounds  
25 capable of overcoming or reversing the inhibition of endothelial cell proliferation. Thus, another aspect of the invention is a screening assay, wherein a nucleic acid, a protein, a peptide or a polypeptide according to the invention is used. For a review of general immunoassays, see e.g. Methods in Cell  
30 Biology, vol. 37: *Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7<sup>th</sup> ed., Stites & Terr,

eds., (1991). The immunoassays may be competitive or non-competitive.

Thus, the present invention also encompasses screening assays aimed at the detection of molecules useful as alternatives to K1-5, which exhibits the same function but are more desirable e-g- due to a smaller molecular

5 weight. Thus, the methods according to the invention are aimed at the detection of peptide or polypeptides, or even proteins, that bind to the same target as K1-5 and therefore exhibit an equivalent therapeutic effect. The peptides or polypeptides, or even proteins detected by the method of the invention are also within the scope of the invention and thus, a further aspect  
10 of the invention is any peptide, polypeptide, or protein that bind to the same receptor as K1-5 as defined by the invention, preferably by SEQ ID NO. 1 and SEQ ID NO 2.

Still another object of the present invention is to provide a diagnostic or  
15 prognostic method, or assay, and kit for detecting the presence and/or amount of the inhibitor in biological fluid samples, such as a body fluid or tissue. In addition, the invention also relates to histochemical kits for localization of the inhibitor defined herein.

20 Another object of the invention is to provide molecular probes to monitor inhibitor biosynthesis and degradation, antibodies that are specific for the inhibitor according to the invention, the development of peptide agonists and antagonists to said inhibitor's receptor, anti-inhibitor receptor-specific antibody agonists and antagonists, and to cytotoxic agents linked to the in-  
25 hibitor.

It is yet another object of the present invention to provide methods and compositions for treating diseases and processes that are mediated by angiogenesis including, but not limited to, hemangioma, solid tumors, leukemia,  
30 metastasis, telangiectasia, psoriasis, scleroderma, pyogenic granuloma, myocardial angiogenesis, plaque neovascularization, coronary collaterals, ce-

rebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, *Helicobacter* related diseases, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, placenta-  
tion, and cat scratch fever.

It is another object of the present invention to provide a composition for treating or repressing the growth of a cancer.

10

It is an object of the present invention to provide compounds that modulate or mimic the production or activity of enzymes that produce the inhibitor of the present invention *in vivo* or *in vitro*.

It is a further object of the present invention to provide the inhibitor or anti-inhibitor antibodies by direct injection of inhibitor DNA into a human or animal needing such treatment.

It is an object of present invention to provide a method for detecting and quantifying the presence of an antibody specific for the inhibitor in a body fluid.

It is another object of the present invention to provide a method for the detection or prognosis of cancer.

25

It is another object of the present invention to provide a composition for use in visualizing and quantitating sites of inhibitor binding *in vivo* and *in vitro*.

It is yet another object of the present invention to provide a composition for use in detection and quantification of inhibitor biosynthesis.

30

It is yet another object of the present invention to provide a therapy for cancer that has minimal side effects, such as gene therapy utilizing a molecule as defined above.

5

Still another object of the present invention is to provide a composition comprising the endothelial cell proliferation inhibitor of the present invention or inhibitor peptide fragment linked to a cytotoxic agent.

10

Another object of the present invention is to provide a method for targeted delivery of inhibitor-related compositions to specific locations.

15

Yet another object of the invention is to provide compositions and methods useful for gene therapy for the modulation of endothelial cell proliferation, such as angiogenic processes.

20

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

#### Detailed Description of the Drawings

*Fig. 1 Proteolytic human K1-5 fragment.*

(A) Kringle 1-5 fragment of human plasminogen was obtained by digestion of Glu<sup>1</sup> plasminogen with urokinase-activated plasmin. The cleavage sites of plasmin is located between the double positively charged Lys76 (77) and Lys77 (78) at the N-terminus, and Arg529 (530) and Lys530 (531) and the C-terminus. The K1-5 fragment containing the region between Lys77-Arg529 was purified by lysine-Sepharose chromatography followed by a Sephadex G-75 column. (B) the purified K1-5 was analyzed on a 10-15%

SDS gradient polyacrylamide gel. The molecular standards in kilodaltons are indicated on the left.

*Fig. 2. Anti-endothelial cell proliferative activity and Inhibition of capillary endothelial cell proliferation.*

5 K1-5 purified from the plasmin digestion of human plasminogen was assayed on bovine capillary endothelial (BCE) cells in the presence of 1 ng/ml bFGF in a 72-h proliferation assay as described previously (Cao et al., J. Biol. Chem 271,29461-29467, 1996; Cao et al., J. Biol. Chem. 272, in  
10 press, 1997). The inhibitory activity of K1-5 on BCE cells was tested at low concentrations (0.2-10 nM) and (B) at high concentration (20 nM-320 nM). (C) The half maximal inhibition of K1-5 on BCE cells was observed at approximately 50 pM. (D) The inhibitory effect is reversible. After removal of  
15 K1-5 from the conditioned medium, endothelial cells are able to proliferate in fresh DMEM medium containing 1 ng/ml of bFGF.

*Fig. 3 Morphology of K1-5-treated endothelial cells.*

In the presence of various concentrations of K1-5, BCE cells stopped proliferation (B-D) as compared to control cells (A). These data suggest that K1-  
20 5 even at high concentrations is not toxic to endothelial cells.

*Fig. 4 Synergistic suppression of endothelial cell growth by angiostatin and K5.*

Pure human angiostatin (K1-4) and kringle 5 (K5) were obtained as proteolytic fragments. These two endothelial cell inhibitors were added either  
25 separately or together to bovine capillary endothelial (BCE) cells stimulated by 1ng/ml basic fibroblast growth factor (bFGF). At the concentration of 1 nM, angiostatin did not exhibit endothelial cell inhibition. Kringle 5 inhibited endothelial cell proliferation by approximately 50%. When angiosta-  
30 tin and K5 were added together to BCE cells, approximately 95% suppres-

sion of cell growth was detected, indicating that both fragments synergistically inhibit endothelial cell proliferation.

5 *Fig. 5. Antiangiogenic effect of K1-5 on the chick embryo chorioallantoic membrane (CAM).*

Methylcellulose disk containing various doses of K1-5 was implanted on CAMs of day 6-old-embryos as described (Cao et al., J. Exp. Med. 182, 2069-2077, 1995).

10 The number of CAMs with avascular zones over the total number of CAMs is indicated as percentages. Phosphate-buffered saline was used as a negative control.

*Fig. 6. Suppression of primary tumor growth by K1-5.*

Approximately,  $1 \times 10^6$  murine T241 fibrosarcoma tumor cells were subcutaneously implanted into each of 6-week-old C57Bl6/J mouse. The mice were systemically  
15 treated with 100  $\mu$ l PBS or 2.5 mg/kg/day K1-5 in 100  $\mu$ l PBS once daily. (A). K1-5-treated mice and a control mouse were photographed at day 20 of treatment. (B). Tumor volumes were measured with a digital calipper and calculated according to a standard formula with x with x length x 0.52. The data represent average mean values (+SEM).

20

*Fig. 7. Detection of microvessel density by immunohistochemical staining of tumor tissue sections with anti-vWF antibodies.*

(A) A control tumor section treated with PBS. (B) and (C) K1-5-treated tumor sections. (D) Quantification of mirovessels of tumor tissue sections per high field.

25

Advantageous Uses of the Invention

In accordance with the present invention, compositions and methods are provided that are effective for inhibiting endothelial cell proliferation, modulating angiogenesis, and inhibiting unwanted angiogenesis, especially an-  
30 giogenesis related to tumor growth. The present invention includes a protein endothelial cell proliferation inhibitor characterized as an approximately

452 amino acid sequence derivable from human plasminogen as kringles 1-5. The amino acid sequence of the inhibitor may vary slightly between species.

5 It is to be understood that the number of amino acids in the active inhibitor molecule may vary and that all closely homologous amino acid sequences that have endothelial inhibiting activity are contemplated as being included in the present invention.

10 The present invention provides methods and compositions for treating diseases and processes mediated by undesired and uncontrolled epithelial cell proliferation, such as angiogenesis, by administering to a human or animal having undesired endothelial cell proliferation a composition comprising approximately kringle 1-5 of human plasminogen capable of inhibiting endothelial cell proliferation in *in vitro* assays. Desirably, the isolated protein  
15 is at least approximately 80% pure, more desirably at least approximately 90% pure and even more desirable at least approximately 90% pure. The present invention is particularly useful for treating, or for repressing the growth of, tumors. Administration of the inhibitor to a human or animal  
20 with prevascularized metastasized tumors helps prevent the growth or expansion of those tumors.

The present invention also encompasses DNA sequences encoding the endothelial cell proliferation inhibitor, expression vectors containing DNA  
25 sequences encoding the endothelial cell proliferation inhibitor, and cells containing one or more expression vectors containing DNA sequences encoding the inhibitor.

The present invention further encompasses gene therapy methods whereby DNA sequences encoding the endothelial cell proliferation inhibitor are introduced into a patient to modify *in vivo* inhibitor levels.  
30

The present invention also includes diagnostic methods and kits for detection and measurement of the endothelial cell proliferation inhibitor in biological fluids and tissues, and for localization of the inhibitor in tissues and cells. The diagnostic method and kit can be in any configuration well known to those of ordinary skill in the art. The present invention also includes antibodies specific for the endothelial cell proliferation inhibitor and portions thereof, and antibodies that inhibit the binding of antibodies specific for the endothelial cell proliferation inhibitor. These antibodies can be polyclonal antibodies or monoclonal antibodies. The antibodies specific for the endothelial cell proliferation inhibitor can be used in diagnostic kits to detect the presence and quantity of the inhibitor which is diagnostic or prognostic for the occurrence or recurrence of cancer or other disease mediated by angiogenesis. Antibodies specific for the endothelial cell proliferation inhibitor may also be administered to a human or animal to passively immunize said human or animal against the inhibitor, thereby reducing angiogenic inhibition.

The present invention also includes diagnostic methods and kits for detecting the presence and quantity of antibodies that bind the endothelial cell proliferation inhibitor in body fluids. The diagnostic method and kit can be in any configuration well known to those of ordinary skill in the art.

The present invention also includes anti-inhibitor receptor-specific antibodies that bind to the inhibitor's receptor and transmit the appropriate signal to the cell and act as agonists or antagonists.

The present invention also includes inhibitor peptide fragments and analogs that can be labeled isotopically or with other molecules or proteins for use in the detection and visualization of the inhibitor binding sites with techniques, including, but not limited to, positron emission tomography, autora-



diography, flow cytometry, radioreceptor binding assays, and immunohistochemistry.

5 These inhibitor peptides and analogs also act as agonists and antagonists at the inhibitor receptor, thereby enhancing or blocking the biological activity of the endothelial cell proliferation inhibitor. Such peptides are used in the isolation of the receptor molecules capable or specifically binding to the inhibitor.

10 The present invention also includes the endothelial cell proliferation inhibitor, inhibitor fragments, antisera specific for the inhibitor, and inhibitor receptor agonists and receptor antagonists linked to cytotoxic agents for therapeutic and research applications.

15 Still further, the inhibitor, fragments thereof, antisera specific therefore, inhibitor receptor agonists and inhibitor receptor antagonists are combined with pharmaceutically acceptable excipients, and optionally sustained-release compounds or compositions, such as biodegradable polymers and matrices, to form therapeutic compositions.

20 The present invention includes molecular probes for the ribonucleic acid and deoxyribonucleic acid involved in transcription and translation of the endothelial cell proliferation inhibitor. These molecular probes are useful for detecting and measuring inhibitor biosynthesis in tissues and cells.

25 The inhibitor may be isolated from plasminogens, such as human plasminogen, or synthesized by chemical or biological methods (e.g. cell culture, recombinant gene expression, peptide synthesis and *in vitro* enzymatic catalysis of plasminogen or plasmin to yield active inhibitor). Recombinant techniques include gene amplification from DNA sources using the polyme-

30

rase chain reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR.

5 The present invention also encompasses a composition comprising a vector containing a DNA sequence encoding the endothelial cell proliferation inhibitor, wherein the vector is capable of expressing the inhibitor when present in a cell, a composition comprising a cell containing a vector, wherein the vector contains a DNA sequence encoding the inhibitor fragments or analogs thereof, and wherein the vector is capable of expressing the inhibitor  
10 when present in the cell, and a method comprising implanting into a human or non-human animal a cell containing a vector, wherein the vector contains a DNA sequence encoding the inhibitor, wherein the vector is capable of expressing the inhibitor when present in the cell.

15 As used herein, the term "substantially similar" or "substantially homologous" when used in reference to the inhibitor amino acid and nucleic acid sequences, means an amino acid sequence having endothelial cell proliferation inhibiting activity and having a molecular weight of approximately 55kDa, which also has a high degree of sequence homology to the protein  
20 having the specific N-terminal amino acid sequence disclosed herein, or a nucleic acid sequence that codes for an endothelial cell proliferation inhibitor having a molecular weight of approximately 55kDa and a high degree of homology to the having the specific N-terminal amino acid sequence disclosed herein.

25 A high degree of homology means at least approximately 80% amino acid homology, desirably at least approximately 90% amino acid homology, and more desirably at least approximately 95% amino acid homology. The term "endothelial inhibiting activity" as used herein means the capability of a  
30 molecule to inhibit angiogenesis in general and, for example, to inhibit the

growth of bovine capillary endothelial cells in culture in the presence of fibroblast growth factor.

5 The present invention also includes the detection of the inhibitor in body fluids and tissues for the purpose of diagnosis or prognosis of diseases such as cancer. The present invention also includes the detection of inhibitor binding sites and receptors in cells and tissues. The present invention also includes methods of treating or preventing angiogenic diseases and processes including, but not limited to, arthritis and tumors by stimulating the production of the inhibitor, and/or by administering isolated inhibitor, or desirable purified inhibitor, or inhibitor agonists or antagonists, and/or inhibitor-specific antisera or antisera directed against inhibitor-specific antisera to a patient. Additional treatment methods include administration of the inhibitor, biologically active fragments thereof, inhibitor analogs, inhibitor-specific antisera, or inhibitor receptor agonists and antagonists linked to  
10  
15 cytotoxic agents.

Passive antibody therapy using antibodies that specifically bind the inhibitor can be employed to modulate angiogenic-dependent processes such as reproduction; development, and wound healing and tissue repair. In addition, antisera directed to the Fab regions of inhibitor-specific antibodies can be administered to block the ability of endogenous inhibitor-specific antisera to bind inhibitor.  
20

25 The present invention also encompasses gene therapy, whereby the gene encoding the inhibitor is regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise rereferred to as gene therapy, are disclosed in Gene Transfer into Mammalian Somatic Cells *in vivo*, N. Yang, Crit. Rev. Biotechn. 12 (4): 335-356 (1992), which is hereby incorporated by reference. Gene therapy encompasses incorporation of DNA sequences into somatic cells or germ line  
30

cells for use in either *ex vivo* or *in vivo* therapy. Gene therapy functions to replace genes, augment normal or abnormal gene function, and to combat infectious diseases and other pathologies.

- 5 Strategies for treating these medical problems with gene therapy include therapeutic strategies, such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene; or prophylactic strategies, such as adding a gene to the product protein that will treat the condition or that will
- 10 make the tissue or organ more susceptible to a treatment regimen. As an example of a prophylactic strategy, a nucleic acid sequence coding for the inhibitor may be placed in a patient and thus prevent occurrence of angiogenesis; or a gene that makes tumor cells more susceptible to radiation could be inserted and then radiation of the tumor would cause increased
- 15 killing of the tumor cells.

Many protocols for transfer of inhibitor DNA or inhibitor regulatory sequences are envisioned in this invention. Transfection of promoter sequences, other than one normally found specifically associated with the

20 inhibitor, or other sequences which would increase production of the inhibitor protein are also envisioned as methods of gene therapy. An example of this technology is found in Transkaryotic Therapies, Inc., of Cambridge, Massachusetts, using homologous recombination to insert a "genetic switch" that turns on an erythropoietin gene in cells. See Genetic Engineering

25 News, April 15, 1994. Such "genetic switches" could be used to activate the inhibitor (or the inhibitor receptor) in cells not normally expressing the inhibitor (or the receptor for the inhibitor).

Gene transfer methods for gene therapy fall into three broad categories -

30 physical (i.e. electroporation, direct gene transfer and particle bombardment), chemical (lipid-based carriers, or other non-viral vectors) and biolo-

gical (virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupffer cells. These cells are long lived and thus provide long term expression of the delivered DNA. Additionally, vectors or the "naked" DNA of the gene may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include *ex vivo* gene transfer, *in vivo* gene transfer and *in vitro* gene transfer. In *ex vivo* gene transfer, cells are taken from the patient and grown in cell culture. The DNA is transfected into the cells, the transfected cells are expanded in number and then reimplanted in the patient. In *in vitro* gene transfer, the transformed cells are cells growing in culture, such as tissue culture cells, and not particular cells from a particular patient. These "laboratory cells" are transfected, the transfected cells are selected and expanded for either implantation into a patient or for other uses.

*In vivo* gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. Methods include using a non-infectious virus to deliver the gene in the patient or injecting naked DNA into a site in the patient and the DNA is taken up by a percentage of cells in which the gene product protein is expressed. Additionally, the other methods described herein, such as use of a "gene gun", may be used for *in vitro* insertion of endothelial cell proliferation inhibitor DNA or inhibitor regulatory sequences.

Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, to ferry the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, make a complex that can cross the cell membrane and provide the DNA into the interior of the cell. Another chemical method uses receptor-based endocytosis, which involves binding a specific ligand to a cell surface receptor and developing and transporting it across the cell membrane. The ligand binds to the DNA and the whole complex is transported into the cell. The ligand gene complex is injected into the blood stream and then target cells that have the receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Many gene therapy methodologies employ viral vectors to insert genes into cells. For example, altered retrovirus vectors have been used in *ex vivo* methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes, or other somatic cells. These altered cells are then introduced into the patient to provide the gene product from the inserted DNA. Viral vectors have also been used to insert genes into cells using *in vivo* protocols. To direct tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue specific can be used. Alternatively, this can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus infected surrounding cells which also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors that have been used for gene therapy protocols include but are not limited to, retroviruses, other RNA viruses such as polio virus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors. Murine leukemia retroviruses are composed of a single strand RNA complexed with a nuclear core protein and polymerase (pol) enzymes, encased by a protein core (gag) and surrounded by a glycoprotein envelope (env) that determines host range. The genomic structure of retroviruses include the gag, pol and env genes enclosed at by the 5' and 3' long terminal repeats (LTR). Retroviral vector systems exploit the fact that a minimal vector containing the 5' and 3' LTRs and the packaging signal are sufficient to allow vector packaging, infection, and integration into target cells providing that the viral structural proteins are supplied in *trans* in the packaging cell line. Fundamental advantages of retroviral vectors for gene transfer include efficient infection and gene expression in most cell types, precise single copy vector into target cell chromosomal DNA, and ease of manipulation of the retroviral genome.

The adenovirus is composed of linear double stranded DNA complexed with core proteins and surrounded with capsid proteins. Advances in molecular virology have led to the ability to exploit the biology of these organisms in order to create vectors capable of transducing novel genetic sequences into target cells *in vivo*. Adenoviral-based vectors will express gene product peptides at high levels. Adenoviral vectors have high efficiencies of infectivity, even with low titers of virus. Additionally, the virus is fully infective as a cell free virion so injection of producer cell lines are not necessary. Another potential advantage to adenoviral vectors is the ability to achieve long term expression of heterologous genes *in vivo*.

Mechanical methods of DNA delivery include fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion, lipid particles of DNA incorporating cationic lipid such as lipofectin, polylysine-mediated transfer of DNA, direct injection of DNA, such as microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles, such as the gold particles used in a "gene gun", and inorganic chemical approaches such as calcium phosphate transfection. Another method, ligand mediated gene therapy, involves complexing the DNA with specific ligands to form ligand-DNA conjugates to direct the DNA to specific cells or tissue.

It has been found that injecting plasmid DNA into muscle cells yields high percentage of the cells which are transfected and have sustained expression of marker genes. The DNA of the plasmid may or may not integrate into the genome of the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions, or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

Particle-mediated gene transfer methods were first used in transforming plant tissue. With a particle bombardment device, or "gene gun", a motive force is generated to accelerate DNA-coated high density particles (such as gold or tungsten) to a high velocity that allows penetration of the target organs, tissues or cells. Particle bombardment can be used in *in vitro* systems, or with *ex vivo* or *in vivo* techniques to introduce DNA into cells, tissues or organs.



Electroporation for gene transfer uses an electrical current to make cells or tissues susceptible to electroporation-mediated gene transfer. A brief electric impulse with a given field strength is used to increase the permeability of a membrane in such a way that DNA molecules can penetrate into the cells. This technique can be used in *in vitro* systems, or with *ex vivo* or *in vivo* techniques to introduce DNA into cells, tissues or organs.

Carrier mediated gene transfer *in vivo* can be used to transfect foreign DNA into cells. The carrier-DNA-complex can be conveniently introduced into body fluids or the bloodstream and then site specifically directed to the target organ or tissue in the body. Both liposomes and polycations, such as polylysine, lipofectins or cytofectins can be used. Liposomes can be developed which are cell specific or organ specific and thus the foreign DNA carried by the liposome will be taken up by target cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing the receptor. Another carrier system that has been used is the asialoglycoprotein/polylysine conjugate system for carrying DNA to hepatocytes for *in vivo* gene transfer.

The transfected DNA may also be complexed with other kinds of carriers so that the DNA is carried to the recipient cell and then resides in the cytoplasm or in the nucleoplasm. DNA can be coupled to carrier nuclear proteins in specifically engineered vesicle complexes and carried directly into the nucleus.

Gene regulation of the inhibitor of the present invention may be accomplished by administering compounds that bind to the gene for the inhibitor, or control regions associated with the gene, or its corresponding RNA transcript to modify the rate of transcription or translation. Additionally, cells transfected with a DNA sequence encoding the inhibitor may be administered to a patient to provide an *in vivo* source of

inhibitor. For example, cells may be transfected with a vector containing a nucleic acid sequence encoding the inhibitor.

5 The term "vector" as used herein means a carrier that can contain or associate with specific nucleic acid sequences, which functions to transport the specific nucleic acid sequences into a cell. Examples of vectors include plasmids and infective microorganisms such as viruses, or non-viral vectors such as ligand-DNA conjugates, liposomes, lipid-DNA complexes. It may be desirable that a recombinant DNA molecule comprising an endothelial cell proliferation inhibitor DNA sequence is operatively linked to an expression control sequence to form an expression vector capable of expressing the inhibitor. The transfected cells may be cells derived from the patient's normal tissue, the patient's diseased tissue, or may be non-patient cells.

15 For example, tumor cells removed from a patient can be transfected with a vector capable of expressing the inhibitor protein of the present invention and re-introduced into the patient. The transfected tumor cells produce levels of inhibitor in the patient that inhibit the growth of the tumor. Patients may be human or non-human animals. Additionally, inhibitor DNA may be directly injected, without the aid of a carrier, into a patient. In particular, inhibitor DNA may be injected into skin, muscle or blood.

20 Inhibitor expression may continue for a long-period of time or inhibitor DNA may be administered periodically to maintain a desired level of the inhibitor protein in the cell, the tissue or organ or biological fluid.

25 Although not wanting to be bound by the following hypothesis, it is believed that when a tumor becomes angiogenic it releases one or more angiogenic peptides (e.g. aFGF, bFGF, VEGF, IL-8, GM-CSF, etc.), which act locally, target endothelium in the neighborhood of a primary tumor from an extravascular direction, and do not circulate (or circulate with a short half-life). These angiogenic peptides must be produced in an amount sufficient to overcome the action of endothelial cell inhibitor

30

(inhibitors of angiogenesis) for a primary tumor to continue to expand its population. Once such a primary tumor is growing well, it continues to release endothelial cell inhibitors into the circulation. According to this hypothesis, these inhibitors act remotely at a distance from the primary tumor, target capillary endothelium of a metastasis from an intravascular direction, and continue to circulate. Thus, just at the time when a remote metastasis might begin to initiate angiogenesis, the capillary endothelium in its neighborhood could be inhibited by incoming inhibitor.

Production of the approximately endothelial cell proliferation inhibitor of the present invention in accomplished using similar techniques can be accomplished using recombinant DNA techniques including the steps of (1) identifying and purifying the inhibitor as described herein and exemplified by the Figures. (2) determining the N-terminal amino acid sequence of the purified inhibitor, (3 ) synthetically generating 5' and 3' DNA oligonucleotide primers for the inhibitor sequence, (4) amplifying the inhibitor gene sequence using polymerase, (5) inserting the amplified sequence into an appropriate vector such as an expression vector, (6) inserting the gene containing vector into a microorganism or other expression system capable of expressing the inhibitor gene, and (7) isolating the recombinantly produced inhibitor. Appropriate vectors include viral, bacterial and eukaryotic (such as yeast) expression vectors. The above techniques are more fully described in laboratory manuals such as *"Molecular Cloning: A Laboratory Manual"* Second Edition by Sambrook et al., Cold Spring Harbor Press, 1989, which is incorporated herein by reference. The contents of all references cited in this application are included herein by reference.

Yet another method of producing the inhibitor, or biologically active fragments thereof, is by peptide synthesis. The amino acid sequence of the inhibitor can be determined, for example by automated peptide sequencing methods. Alternatively, once the gene or DNA sequence which codes for inhibitor is isolated, for example by the methods described above, the DNA sequence can be determined using manual

or automated sequencing methods well known in the art. The nucleic acid sequence in turn provides information regarding the amino acid sequence.

5 Once the amino acid sequence of the peptide is known, peptide fragments can be synthesized by techniques well known in the art, as exemplified by "Solid Phase Peptide Synthesis: A Practical Approach" E. Atherton and R.C. Sheppard. IRL Press, Oxford, England. Multiple fragments can be synthesized which are subsequently linked together to form larger fragments. These synthetic peptide fragments can also be made with amino acid substitutions at specific locations in order  
10 to test for agonistic and antagonistic activity *in vitro* and *in vivo*. Peptide fragments that possess high affinity binding to tissues can be used to isolate receptors that bind the inhibitor on affinity columns.

15 The inhibitor is effective in treating diseases or processes such as angiogenesis, that are mediated by, or involve, endothelial cell proliferation. The present invention includes the method of treating an angiogenesis mediated disease with an effective amount of inhibitor, or a biologically active fragment thereof, or combinations of inhibitor fragments that collectively possess anti-angiogenic activity or inhibitor agonists and antagonists. The angiogenesis mediated diseases include, but are not  
20 limited to, solid tumors; blood born tumors such as leukemias; tumor metastasis; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia,  
25 rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophilic joints; angiofibroma; and wound granulation.

The inhibitor is useful in the treatment of diseases of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal  
30 adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. The inhibitor can be used as a birth control agent by preventing vascularization required

for embryo implantation. The inhibitor is useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochelium minalia quintosa*) and ulcers (*Helicobacter pylori*).

- 5     The synthetic peptide fragments of the inhibitor have a variety of uses. The peptide that binds to receptor capable of binding the inhibitor with high specificity and avidity is radiolabeled and employed for visualization and quantitation of binding sites using autoradiographic and membrane binding techniques.
- 10    In addition, labeling inhibitor or peptide fragments thereof with short lived isotopes enables visualization of receptor binding sites *in vivo* using positron emission tomography or other modern radiographic techniques in order to locate tumors with inhibitor binding sites.
- 15    Cytotoxic agents, such as ricin, are linked to the inhibitor, and high affinity peptide fragments thereof, thereby providing a tool for destruction of cells that bind the inhibitor. These cells may be found in many locations, including but not limited to, micrometastases and primary tumors. Peptides linked to cytotoxic agents are infused in a manner designed to maximize delivery to the desired location. For example,
- 20    delivery may be accomplished through a cannula into vessels supplying the target site or directly into the target. Such agents are also delivered in a controlled manner through osmotic pumps coupled to infusion cannulae. A combination of inhibitor antagonists may be co-applied with stimulators of angiogenesis to increase vascularization of tissue. This therapeutic regimen provides an effective means of destroying
- 25    metastatic cancer.

- The inhibitor may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy combined with the inhibitor and then the
- 30    inhibitor may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tu-

mor. Additionally, the inhibitor, fragments thereof, inhibitor-specific antisera, inhibitor receptor agonists and antagonists, or combinations thereof, are combined with pharmaceutically acceptable excipients, and optionally sustained-release matrix, such as biodegradable polymers, to form therapeutic compositions.

5

A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid/base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. The sustained-release matrix desirably is chosen from biocompatible materials, such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (co-polymers of lactic acid and glycolic acid), polyanhydrides, poly(ortho)esters, polypeptides, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid).

10

15

20

25

The angiogenesis-modulating therapeutic composition of the present invention may be a solid, liquid or aerosol and may be administered by any known route of administration. Examples of solid therapeutic compositions include pills, creams, and implantable dosage units. The pills may be administered orally, the therapeutic creams may be administered topically. The implantable dosage units may be administered locally, for example at a tumor site, or which may be implanted for systemic release of the therapeutic angiogenesis modulating composition, for example subcutaneously. Examples of liquid composition include formulations adapted for injection subcutaneously, intravenously, intraarterially and formulations for topical and intraocular administration. Examples of aerosol formulations include inhaler formulation for administration to the lungs.

30

The inhibitor protein of the present invention also can be used to generate antibodies that are specific for the inhibitor and its receptor. The antibodies can be either polyclonal antibodies or monoclonal antibodies. These antibodies that specifically bind to the inhibitor or inhibitor receptors can be used in diagnostic methods and kits that are well known to those of ordinary skill in the art to detect or quantify the inhibitor levels or inhibitor receptors levels in a body fluid or tissue. Results from these tests can be used to diagnose or predict the occurrence or recurrence of a cancer and other angiogenic mediated diseases.

The inhibitor also can be used to develop a diagnostic method and kit to detect and quantify antibodies capable of binding the inhibitor. These kits would permit detection of circulating inhibitor-specific antibodies. Patients that have such circulating anti-inhibitor antibodies may be more likely to develop multiple tumors and cancers, and may be more likely to have recurrences of cancer after treatments or periods of remission. The Fab fragments of these antibodies may be used as antigens to generate anti-inhibitor-specific Fab-fragment antisera which can be used to neutralize anti-inhibitor antibodies. Such a method would reduce the removal of circulating inhibitor by anti-inhibitor antibodies, thereby effectively elevating circulating inhibitor levels.

Another aspect of the present invention is a method of blocking the action of excess endogenous inhibitor. This can be done by passively immunizing a human or animal with antibodies specific for the undesired inhibitor in the system. This treatment can be important in treating abnormal ovulation, menstruation and placentation, and vasculogenesis. This provides a useful tool to examine the effects of inhibitor removal on metastatic processes. The Fab fragment of inhibitor-specific antibodies contains the binding site for inhibitor. This fragment is isolated from inhibitor-specific antibodies using techniques known to those skilled in the art. The Fab fragment of inhibitor-specific antisera are used as antigens to generate production of anti-Fab fragment serum. Infusion of this antiserum against the Fab fragments specific for the inhibitor prevents the inhibitor from binding to inhibitor antibodies. Therapeutic be-

nefit is obtained by neutralizing endogenous anti-inhibitor antibodies by blocking the binding of inhibitor to the Fab fragments or anti-inhibitor. The net effect of this treatment is to facilitate the ability of endogenous circulating inhibitor to reach target cells, thereby decreasing the spread of metastases.

5

It is to be understood that the present invention is contemplated to include any derivatives of the inhibitor that have endothelial cell proliferation inhibitor activity. The present invention includes the entire inhibitor protein, derivatives of the inhibitor protein and biologically-active fragments of the inhibitor protein. These include  
10 proteins with inhibitor activity that have amino acid substitutions or have sugars or other molecules attached to amino acid functional groups. The present invention also includes genes that code for the inhibitor and the inhibitor receptor, and to proteins that are expressed by those genes.

15 The proteins and protein fragments with the inhibitor activity described above can be provided as isolated and substantially purified proteins and protein fragments in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, trans-  
20 dermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, the inhibitor may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example,  
25 at the site of a tumor or implanted so that the inhibitor is slowly released systemically. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of the inhibitor through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor. The biodegradable polymers and their use are described, for example, in detail in Brem et al.,  
30 J. Neurosurg. 74:441-446 (1991), which is hereby incorporated by reference in its entirety.



The dosage of the inhibitor of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating humans or animals, approximately 5 mg/kg/day, administered once a day, suppresses tumor growth to >50%. In the same dosages, angiostatin does not have any effect. Depending upon the half-life of the inhibitor in the particular animal or human, the inhibitor can be administered between several times per day to once a week. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The inhibitor formulations include those suitable for oral, rectal, ophthalmic (including intravitreal or intracameral, nasal, topical (including buccal and sublingual), intrauterine, vaginal or parenteral (including subcutaneous, intraperitoneal, intramuscular, intravenous, intradermal, intracranial, intratracheal, and epidural) administration. The inhibitor formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presen-

ted in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from  
5 sterile powders, granules and tablets of the kind previously described. Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard  
10 to the type of formulation in question. Optionally, cytotoxic agents may be incorporated or otherwise combined with inhibitor proteins, or biologically functional peptide fragments thereof, to provide dual therapy to the patient.

Angiogenesis inhibiting peptides of the present invention can be synthesized in a  
15 standard microchemical facility and purity checked with HPLC and mass spectrophotometry. Methods of peptide synthesis, HPLC purification and mass spectrophotometry are commonly known to those skilled in these art. Inhibitor peptides and inhibitor receptors peptides are also produced in recombinant E. coli or yeast expression systems, and purified with column chromatography.

20 Different peptide fragments of the intact inhibitor molecule can be synthesized for use in several applications including, but not limited to the following; as antigens for the development of specific antisera, as agonists and antagonists active at inhibitor binding sites, as peptides to be linked to, or used in combination with, cytotoxic agents for targeted killing of cells that bind the inhibitor. The amino acid  
25 sequences that comprise these peptides are selected on the basis of their position on the exterior regions of the molecule and are accessible for binding to antisera. The amino and carboxyl termini of the inhibitor, as well as the mid-region of the molecule are represented separately among the fragments to be synthesized.

30

These peptide sequences are compared to known sequences using protein sequence databases such as GenBank, Brookhaven Protein, SWISS-PROT, and PIR to determine potential sequence homologies. This information facilitates elimination of sequences that exhibit a high degree of sequence homology to other molecules, thereby enhancing the potential for high specificity in the development of antisera, agonists and antagonists to the inhibitor.

Inhibitor and inhibitor derived peptides can be coupled to other molecules using standard methods. The amino and carboxyl termini of the inhibitor both contain tyrosine and lysine residues and are isotopically and nonisotopically labeled with many techniques, for example radiolabeling using conventional techniques (tyrosine residues- chloramine T, iodogen, lactoperoxidase; lysine residues- Bolton-Hunter reagent). These coupling techniques are well known to those skilled in the art. Alternatively, tyrosine or lysine is added to fragments that do not have these residues to facilitate labeling of reactive amino and hydroxyl groups on the peptide. The coupling technique is chosen on the basis of the functional groups available on the amino acids including, but not limited to amino, sulfhydryl, carboxyl, amide, phenol and imidazole. Various reagents used to effect these couplings include among others, glutaraldehyde, diazotized benzidine, carbodiimide, and p-benzoquinone. Inhibitor peptides are chemically coupled to isotopes, enzymes, carrier proteins, cytotoxic agents, fluorescent molecules, chemiluminescent, bioluminescent and other compounds for a variety of applications. The efficiency of the coupling reaction is determined using different techniques appropriate for the specific reaction. For example, radiolabeling of an inhibitor peptide with  $^{125}\text{I}$  is accomplished using chloramine T and  $\text{Na}^{125}\text{I}$  of high specific activity. The reaction is terminated with sodium metabisulfite and the mixture is desalted on disposable columns. The labeled peptide is eluted from the column and fractions are collected. Aliquots are removed from each fraction and radioactivity measured in a gamma counter. In this manner, the unreacted  $\text{Na}^{125}\text{I}$  is separated from the labeled inhibitor peptide. The peptide fractions with the highest specific radioactivity are stored for subsequent use such as analysis of the ability to bind to inhibitor antisera.

Another application of peptide conjugation is for production of polyclonal antisera. For example, inhibitor peptides containing lysine residues are linked to purified bovine serum albumin using glutaraldehyde. The efficiency of the reaction is determined by measuring the incorporation of radiolabeled peptide. Unreacted glutaraldehyde and peptide are separated by dialysis. The conjugate is stored for subsequent use.

Antiserum specific for the inhibitor, inhibitor analogs, peptide fragments of the inhibitor and the inhibitor receptor can be generated. After peptide synthesis and purification, both monoclonal and polyclonal antisera are raised using established techniques known to those skilled in the art. For example, polyclonal antisera may be raised in rabbits, sheep, goats or other animals. Inhibitor peptides conjugated to a carrier molecule such as bovine serum albumin, or inhibitor itself, is combined with an adjuvant mixture, emulsified and injected subcutaneously at multiple sites on the back, neck, flanks, and sometimes in the rootpads. Booster injections are made at regular intervals, such as every 2 to 4 weeks. Blood samples are obtained by venipuncture, for example using the marginal ear veins after dilation, approximately 7 to 10 days after each injection. The blood samples are allowed to clot overnight at 4°C and are centrifuged at approximately 2400 X g at 4°C for about 30 minutes. The serum is removed, aliquoted, and stored at 4°C for immediate use for subsequent analysis.

All serum samples from generation of polyclonal antisera or media samples from production of monoclonal antisera are analyzed for determination of antibody titer. Titer is established through several means, for example, using dot blots and density analysis, and also with precipitation of radiolabeled peptide-antibody complexes using protein A, secondary antisera, cold ethanol or charcoal-dextran followed by activity measurement with a gamma counter. The highest titer antisera are also purified on affinity columns which are commercially available. Inhibitor peptides are coupled to the gel in the affinity column. Antiserum samples are passed through the

column and anti-inhibitor antibodies remain bound to the column. These antibodies are subsequently eluted, collected and evaluated for determination of titer and specificity.

- 5 The highest titer inhibitor-specific antisera is tested to establish the following; a) optimal antiserum dilution for highest specific binding of the antigen and lowest non-specific binding, b) the ability to bind increasing amounts of inhibitor peptide in a standard displacement curve, c) potential cross-reactivity with related peptides and proteins of related species, d) ability to detect inhibitor peptides in extracts of  
10 plasma, urine, tissues and in cell culture media.

Kits for measurement of inhibitor, and the inhibitor receptor, are also contemplated as part of the present invention. Antisera that possess the highest titer and specificity and can detect inhibitor peptides in extracts of plasma, urine, tissues, and in cell  
15 culture media are further examined to establish easy to use kits for rapid, reliable, sensitive, and specific measurement and localization of inhibitor. These assay kits include but are not limited to the following techniques; competitive and non-competitive assays, radioimmunoassay, bioluminescence and hemiluminescence assay, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, microtiter plates, antibody coated strips or  
20 dipsticks for rapid monitoring of urine or blood, and immunocytochemistry. For each kit the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established. Intraassay and interassay variation is established at 20%, 50% and 80% points on the standard curves of displacement or activity.

25 One example of an assay kit commonly used in research and in the clinic is a radioimmunoassay (RIA) kit. An inhibitor RIA is illustrated below. After successful radioiodination and purification of inhibitor or an inhibitor peptide, the antiserum possessing the highest titer is added at several dilutions to tubes containing a relatively constant amount of radioactivity, such as 10,000 cpm, in a suitable buffer system. Other tubes contain buffer or preimmune serum to determine the non-specific  
30

binding. After incubation at 4°C for 24 hours, protein A is added and the tubes are vortexed, incubated at room temperature for 90 minutes and centrifuged at approximately 2000 - 2500 X at 4°C to precipitate the complexes of antibody bound to labeled antigen. The supernatant is removed by aspiration and the radioactivity in the pellets counted in gamma counter. The antiserum dilution that binds approximately 10% to 40% of the labeled peptide after subtraction of the non-specific binding is further characterized.

Next, a dilution range (approximately 0,1 pg to 10 ng) of the inhibitor peptide used for development or the antiserum is evaluated by adding known amounts of the peptide to tubes containing radiolabeled peptide and antiserum. After an additional incubation period, for example, 24 to 48 hours, protein A is added and the tubes centrifuged, supernatant removed and the radioactivity in the pellet counted. The displacement of the binding of radiolabeled inhibitor peptide by the unlabeled inhibitor peptide (standard) provides a standard curve. Several concentrations of other inhibitor peptide fragments, inhibitor from different species, and homologous peptides are added to the assay tubes to characterize the specificity of the inhibitor antiserum.

Extracts of various tissues, including but not limited to primary and secondary tumors, Lewis lung carcinoma, cultures of inhibitor producing cells, placenta, uterus, and other tissues such as brain, liver, and intestine, are prepared. After lyophilization or Speed Vac of the tissue extracts, assay buffer is added and different aliquots are placed into the RIA tubes. Extracts of inhibitor producing cells produce displacement curves that are parallel to the standard curve, whereas extracts of tissues that do not produce inhibitor do not displace radiolabeled inhibitor from the inhibitor. In addition, extracts of urine, plasma, and cerebrospinal fluid from animals with Lewis lung carcinoma are added to the assay tubes in increasing amounts. Parallel displacement curves indicate the utility of the inhibitor assay to measure inhibitor in tissues and body fluids.

Tissue extracts that contain inhibitor are additionally characterized by subjecting aliquots to reverse phase HPLC. Eluate fractions are collected, dried in Speed Vac, reconstituted in RIA buffer and analyzed in the inhibitor RIA. The maximal amount of inhibitor immunoreactivity is located in the fractions corresponding to the elution position of inhibitor.

The assay kit provides instructions, antiserum, inhibitor or inhibitor peptide and possible radiolabeled inhibitor and/or reagents for precipitation of bound inhibitor-inhibitor antibody complexes. The kit is useful for the measurement of inhibitor in biological fluids and tissue extracts of animals and humans with and without tumors.

Another kit is used for localization of inhibitor in tissues and cells. This inhibitor immunohistochemistry kit provides instructions, inhibitor antiserum, and possibly blocking serum and secondary antiserum linked to a fluorescent molecule such as fluorescein isothiocyanate, or to some other reagent used to visualize the primary antiserum. Immunohistochemistry techniques are well known to those skilled in the art. This inhibitor immunohistochemistry kit permits localization of inhibitor in tissue sections and cultured cells using both light and electron microscopy. It is used for both research and clinical purposes. For example, tumors are biopsied or collected and tissue sections cut with a microtome to examine sites of inhibitor production. Such information is useful for diagnostic and possibly therapeutic purposes in the detection and treatment of cancer. Another method to visualize sites of inhibitor biosynthesis involves radiolabeling nucleic acids for use in *in situ* hybridization to probe for inhibitor messenger RNA. Similarly, the inhibitor receptor can be localized, visualized and quantitated with immunohistochemistry techniques.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other em-

bodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art.



## EXAMPLE 1

### Methods

#### 5    1. Preparation of kringle 1-5 (K1-5) from human plasminogen

Human plasminogen was prepared from pooled human plasma by lysine-Sephacryl chromatography as previously described (Cao et al., 1996, JBC, 271, 29461-29467). Purified human plasminogen was incubated in an alkaline solution. The digested mixture was loaded in a lysine-Sephacryl column and K1-5 was eluted with a 0-25  $\epsilon$ -aminocaproic acid gradient. The K1-5 fraction was further purified on a Sephacryl-S200 column (2,5 x 70 to remove trace amounts of plasminogen contamination. The molecular weight was 55, 000 Dalton verified on SDS gel electrophoresis. Microsequencing analysis revealed that K1-5 consists of a fragment corresponding to plasminogen Lys 77-Arg 529.

10

15

#### 2. Endothelial cell proliferation assay

Bovine capillary endothelial cells were maintained in DMEM with 10% heat-inactivated BCS, antibiotics and 3 ng/ml recombinant human bFGF.

20    Cells were washed with PBS and dispersed in a 0,05% solution of trypsin. A cell suspension was made with DMEM, 10% BCS, 1% antibiotics and the concentration was adjusted to 25,000 cells/ml after hemocytometer count. Cells were plated onto gelatinized 24-well culture plates (0,5 ml/well) and were incubated (37°C in 10% CO<sub>2</sub>) for 14h. The medium was replaced with

25    0,25 ml DMEM, 5% BCS and the test samples at different concentrations were applied. After 20 min incubation, medium and bFGF were added to each well to obtain a final volume of 0,5 ml of DMEM, 5% BCS, 1% antibiotics and 1 ng/ml bFGF. After 72h incubation, cells were dispersed in trypsin, resuspended in Hematall and counted by Coulter counter.

## EXAMPLE 2

### 1. Preparation of K1-5 from human plasminogen:

5 Human plasminogen was prepared from pooled human plasma by lysine-Sepharose chromatography as previously described (Cao et al., 1996, JBC, 271, 29461, 29467). approximately 40 mg of plasminogen in 4 ml of glycine buffer (pH 10.5) was incubated with immobilized urokinase-activated plasmin (10 Mol of plasminogen / 1 Mol plasmin) for 24 h at 25°C. The digested mixture was loaded in a lysine-Sepharose column (1.0 x 30 cm), and K1-5 was eluted with a 0-25  $\epsilon$ -aminocaproic acid gradient. The K1-5 fraction was further purified on a Sephadex G-75 column (2.6 x 90). The molecular weight was 55,000 verified on SDS gel electrophoresis. Microsequencing analysis revealed that K1-5 consists of a fragment corresponding to plasminogen Lys 77 (78) - Arg 529 (530).

### 2. Endothelial cell proliferation assay:

Bovine capillary endothelial cells were maintained in DMEM with 10% heat-inactivated BCS, antibiotics and 3 ng/ml recombinant human bFGF. Cells were washed with PBS and were dispersed in a 0.05% solution of trypsin. A cell suspension was made with DMEM, 10% BCS, 1% antibiotics and the concentration was adjusted to 25,000 cells/ml after hemocytometer count. Cells were plated onto gelatinized 24-well culture plates (0.5 ml/well) and were incubated (37°C in 10% CO<sub>2</sub>) for 14 h. The medium was replaced with 0.25 ml DMEM, 5% BCS and the test samples at different concentrations were applied. After 20 min incubation, medium and bFGF were added to each well to obtain a final volume of 0.5 ml of DMEM, 5% BCS, 1% antibiotics and 1 ng/ml bFGF. After 72 h incubation, cells were

dispersed in trypsin, resuspended in Hematall and counted by Coulter counter.

### 3. Chick embryo chorioallantoic membrane assay (CAM):

5

3-d-old fertilized white Leghorn eggs were cracked, and chick embryos with intact yolks were placed in 100 x 20 mm plastic petri dishes. After 3-d of incubation in 3% CO<sub>2</sub> at 37°C, a disk of methylcellulose containing 10 µg of K1-5 was implanted on the CAM of each individual embryo. After 48 h incubation, embryos and CAMs were analyzed for the formation of avascular zones by a stereoscope.

10

### 4. Tumor studies in mice:

15

Male 6-week-old C57B16/J mice were used for tumor studies. Murine T241 fibrosarcoma cells ( $1 \times 10^6$ ) growing in a log phase were harvested, resuspended in PBS, and implanted subcutaneously in the midline dorsum of each animal in a volume of 100 µl. Three mice were used in each treatment and control group. Subcutaneous injections with either 100 µl of PBS or 100 µl of K1-5 were begun shortly after injection of tumor cells and continued once a day, for a total of 20 days. Visible tumors were present after 24 h. Primary tumors were present after 24 h. Primary tumors were measured blindly using digital calipers on the days indicated.

20

25

SEQUENCE LISTINGSEQ ID NO. 1Amino acid sequence of Human Kringle 1-5 (453 amino acids):

Lys Val Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly Lys Asn Tyr Arg Gly Thr Met Ser Lys Thr  
Lys Asn Gly Ile Thr Cys Gln Lys Trp Ser Ser Thr Ser Pro His Arg Pro Arg Phe Ser Pro Ala Thr  
His Pro Ser Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Pro Gln Gly Pro Trp  
Cys Tyr Thr Thr Asp Pro Glu Lys Arg Tyr Asp Tyr Cys Asp Ile Leu Glu Cys Glu Glu Cys  
Met His Cys Ser Gly Glu Asn Tyr Asp Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Glu Cys Gln Ala  
Trp Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro Asn Lys Asn Leu Lys Lys  
Asn Tyr Cys Arg Asn Pro Asp Arg Glu Leu Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys Arg  
Trp Glu Leu Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Ser Ser Gly Pro Thr Tyr Gln Cys Leu  
Lys Gly Thr Gly Glu Asn Tyr Arg Gly Asn Val Ala Val Thr Val Ser Gly His Thr Cys Gln His Trp  
Ser Ala Gln Thr Pro His Thr His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu  
Asn Tyr Cys Arg Asn Pro Asp Gly Lys Arg Ala Pro Trp Cys His Thr Thr Asn Ser Gln Val Arg  
Trp Glu Tyr Cys Lys Ile Pro Ser Cys Asp Ser Ser Pro Val Ser Thr Glu Gln Leu Ala Pro Thr Ala  
Pro Pro Glu Leu Thr Pro Val Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser Tyr Arg Gly Thr Ser  
Ser Thr Thr Thr Thr Gly Lys Lys Cys Gln Ser Trp Ser Ser Met Thr Pro His Arg His Gln Lys Thr  
Pro Glu Asn Tyr Pro Asn Ala Gly Leu Thr Met Asn Tyr Cys Arg AsnPro Asp Ala Asp Lys Gly  
Pro Trp Cys Phe Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Lys Lys Cys Ser Gly  
Thr Glu Ala Ser Val Val Ala Pro Pro Pro Val Val Leu Leu Pro Asp Val Glu Thr Pro Ser Glu Glu  
Asp Cys Met Phe Gly Asn Gly Lys Gly Tyr Arg Gly Lys Arg Ala Thr Thr Val Thr Gly Thr Pro  
Cys Gln Asp Trp Ala Ala Gln Glu Pro His Arg His Ser Ile Phe Thr Pro Glu Thr Asn Pro Arg Ala  
Gly Leu Glu Lys Asn Tyr Cys Arg Asn Pro Asp Gly Asp Val Gly Gly Pro Trp Cys Tyr Thr Thr  
Asn Pro Arg

SEQ ID NO. 2**Amino acid sequence of mouse Kringle 1-5 (453 amino acids):**

Arg Val Tyr Leu Ser Glu Cys Lys Thr Gly Ile Gly Asn Gly Tyr Arg Gly Thr Met Ser Arg Thr Lys  
Ser Gly Val Ala Cys Gln Lys Trp Gly Ala Thr Phe Pro His Val Pro Asn Tyr Ser Pro Ser Thr His  
Pro Asn Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Gln Gly Pro Trp Cys  
Tyr Thr Thr Asp Pro Asp Lys Arg Tyr Asp Tyr Cys Asn Ile Pro Glu Cys Glu Glu Glu Cys Met  
Tyr Cys Ser Gly Glu Lys Tyr Glu Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Asp Cys Gln Ala Trp  
Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ala Lys Phe Pro Ser Lys Asn Leu Lys Met Asn  
Tyr Cys His Asn Pro Asp Gly Glu Pro Arg Pro Trp Cys Phe Thr Thr Asp Pro Thr Lys Arg Trp  
Glu Tyr Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Ser Pro Thr Tyr Gln Cys Leu Lys  
Gly Arg Gly Glu Asn Tyr Arg Gly Thr Val Ser Val Thr Val Ser Gly Lys Thr Cys Gln Arg Trp Ser  
Glu Gln Thr Pro His Arg His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Glu Glu Asn  
Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr Thr Asp Ser Gln Leu Arg Trp  
Glu Tyr Cys Glu Ile Pro Ser Cys Glu Ser Ser Ala Ser Pro Asp Gln Ser Asp Ser Ser Val Pro Pro  
Glu Glu Gln Thr Pro Val Val Gln Glu Cys Tyr Gln Ser Asp Gly Gln Ser Tyr Arg Gly Thr Ser Ser  
Thr Thr Ile Thr Gly Lys Lys Cys Gln Ser Trp Ala Ala Met Phe Pro His Arg His Ser Lys Thr Pro  
Glu Asn Phe Pro Asp Ala Gly Leu Glu Met Asn Tyr Cys Arg Asn Pro Asp Gly Asp Lys Gly Pro  
Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Lys Arg Cys Ser Glu Thr  
Gly Gly Ser Val Val Glu Leu Pro Thr Val Ser Gln Glu Pro Ser Gly Pro Ser Asp Ser Glu Thr Asp  
Cys Met Tyr Gly Asn Gly Lys Asp Tyr Arg Gly Lys Thr Ala Val Thr Ala Ala Gly Thr Pro Cys  
Gln Gly Trp Ala Ala Gln Glu Pro His Arg His Ser Ile Phe Thr Pro Gln Thr Asn Pro Arg Ala Asp  
Leu Glu Lys Asn Tyr Cys Arg Asn Pro Asp Gly Asp Val Asn Gly Pro Trp Cys Tyr Thr Thr Asn  
Pro Arg

CLAIMS

1. An isolated protein capable of modulating endothelial cell proliferation  
*in vitro* and angiogenesis *in vivo* and having an amino acid comprising at  
5 least about 50% of the sequence disclosed in SEQ ID NO. 1 or SEQ ID  
NO. 2 or an analogue thereof.
2. A protein according to claim 1, or a functional fragment or an analogue  
thereof, comprising the sequence from Lys 78-Arg 530 of a plasminogen  
10 molecule.
3. A protein according to claim 1 or 2, or a functional fragment or an analo-  
gue thereof, having a molecular weight of between about 50 and about 60,  
preferably about 53 -57, and most preferably about 55 kilodalton, as deter-  
15 mined by polyacrylamide gel electrophoresis.
4. A protein according to any one of claims 1-3, or a functional fragment or  
an analogue thereof, which is capable of inhibiting angiogenesis.
- 20 5. A protein according to any one of claims 1-4, or a functional fragment or  
an analogue thereof, which comprises essentially all of the amino acid  
sequence disclosed in SEQ ID NO. 1 and is of human origin.
- 25 6. A protein according to any one of claims 1-4, or a functional fragment or  
an analogue thereof, which comprises essentially all of the amino acid  
sequence disclosed in SEQ ID NO. 2 and is of mouse origin.
7. Use of a protein according to any one of claims 1-6, or a functional frag-  
ment or an analogue thereof, as a medicament.

8. Use of a protein according to any one of claims 1-6, or a functional fragment or an analogue thereof, for the manufacture of a medicament for the treatment of angiogenesis associated diseases or disorders, such as cancer or diabetes.
- 5
9. A pharmaceutical or veterinary composition comprising a protein according to any one of claims 1-6, or a functional fragment or an analogue thereof, as well as one or more pharmaceutically acceptable carriers and/or excipients.
- 10
10. A nucleic acid, which encodes a protein according to any one of claims 1-6 or a functional fragment or an analogue thereof, or any variant thereof.
- 15
11. A nucleic acid, which specifically hybridizes, under stringent conditions, to a nucleic acid according to claim 10.
12. A peptide, polypeptide or protein encoded by a nucleic acid according to claim 11, or a functional fragment or an analogue thereof.
- 20
13. An antibody raised against a peptide, polypeptide or protein according to claim 12.
14. A screening assay, wherein a nucleic acid according to claim 11 or 11, a peptide, polypeptide or protein according to claim 12 or an antibody according to claim 13 is used.
- 25
15. A method of diagnosis and/or prognosis, wherein a nucleic acid according to claim 11 or 11, a peptide, polypeptide or protein according to claim 12 or an antibody according to claim 13 is used.
- 30

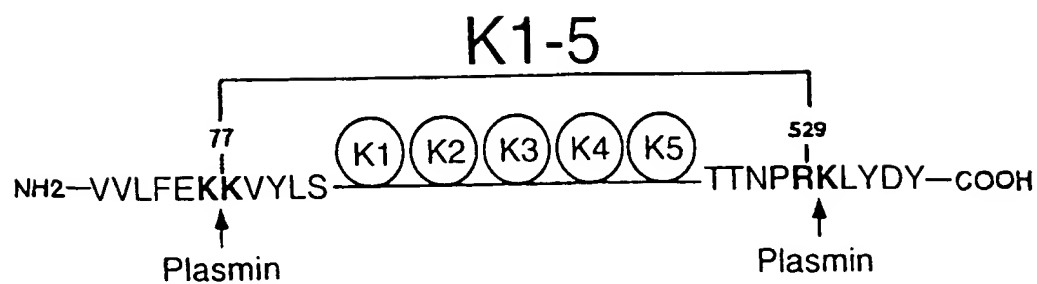
16. A kit for performing a method according to claim 15.



**Fig. 1**

**1/8**

A



B

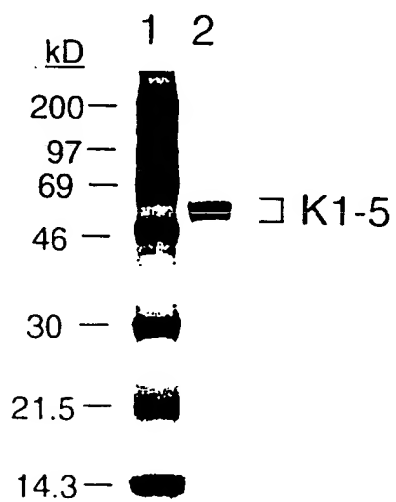


Fig. 2

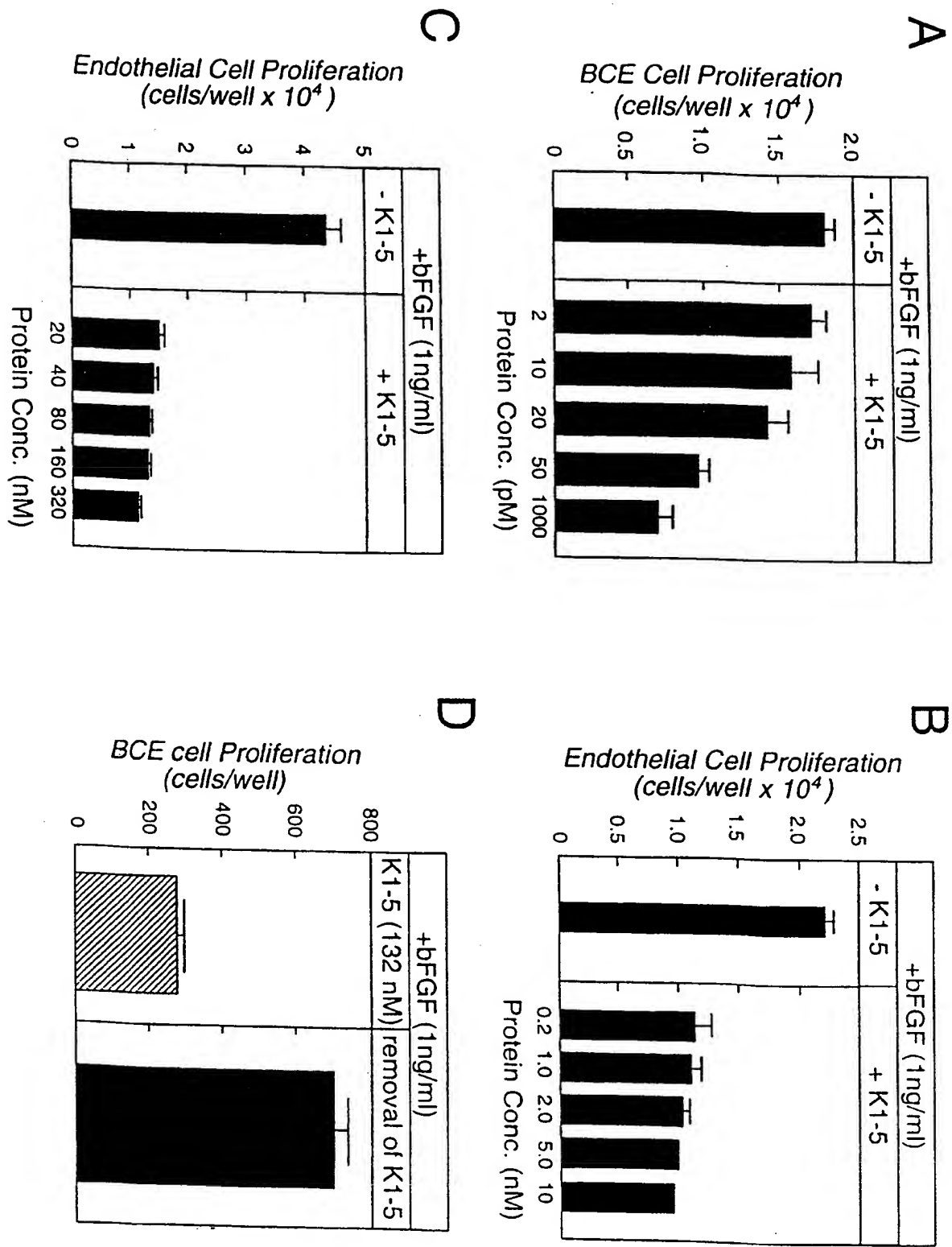


Fig. 3

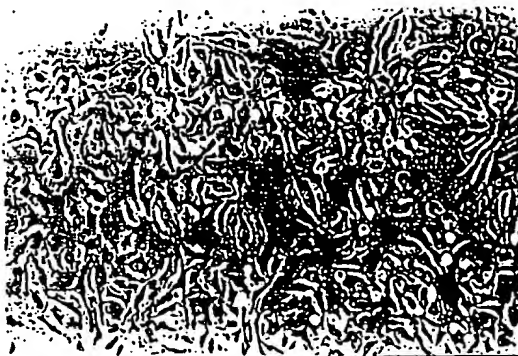
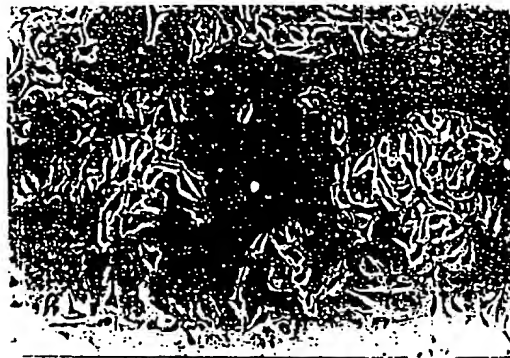
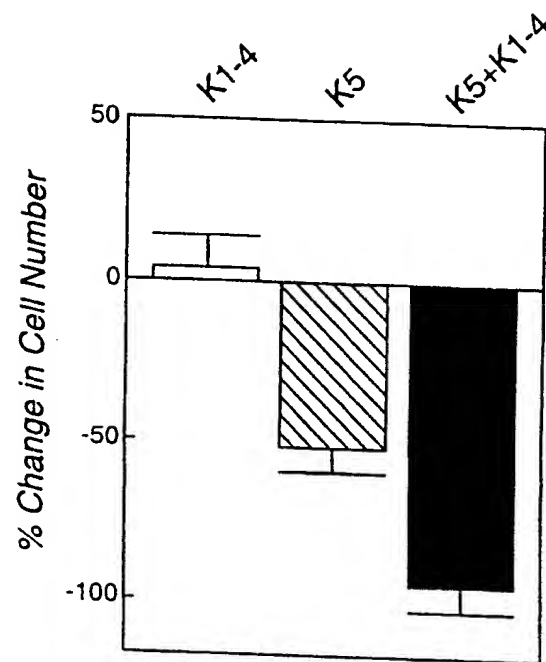
**A** bFGF alone**B** bFGF + K1-5 (10ng/ml)**C** bFGF + K1-5 (100ng/ml)**D** bFGF + K1-5 (500ng/ml)

Fig. 4

4/8



5/8

Fig. 5

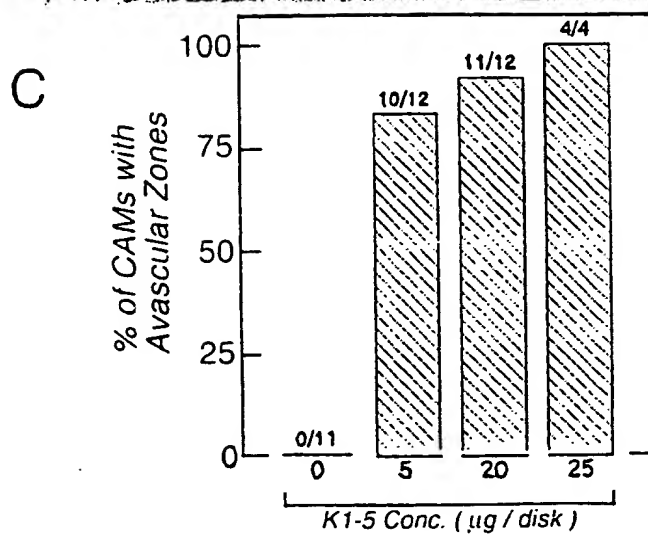
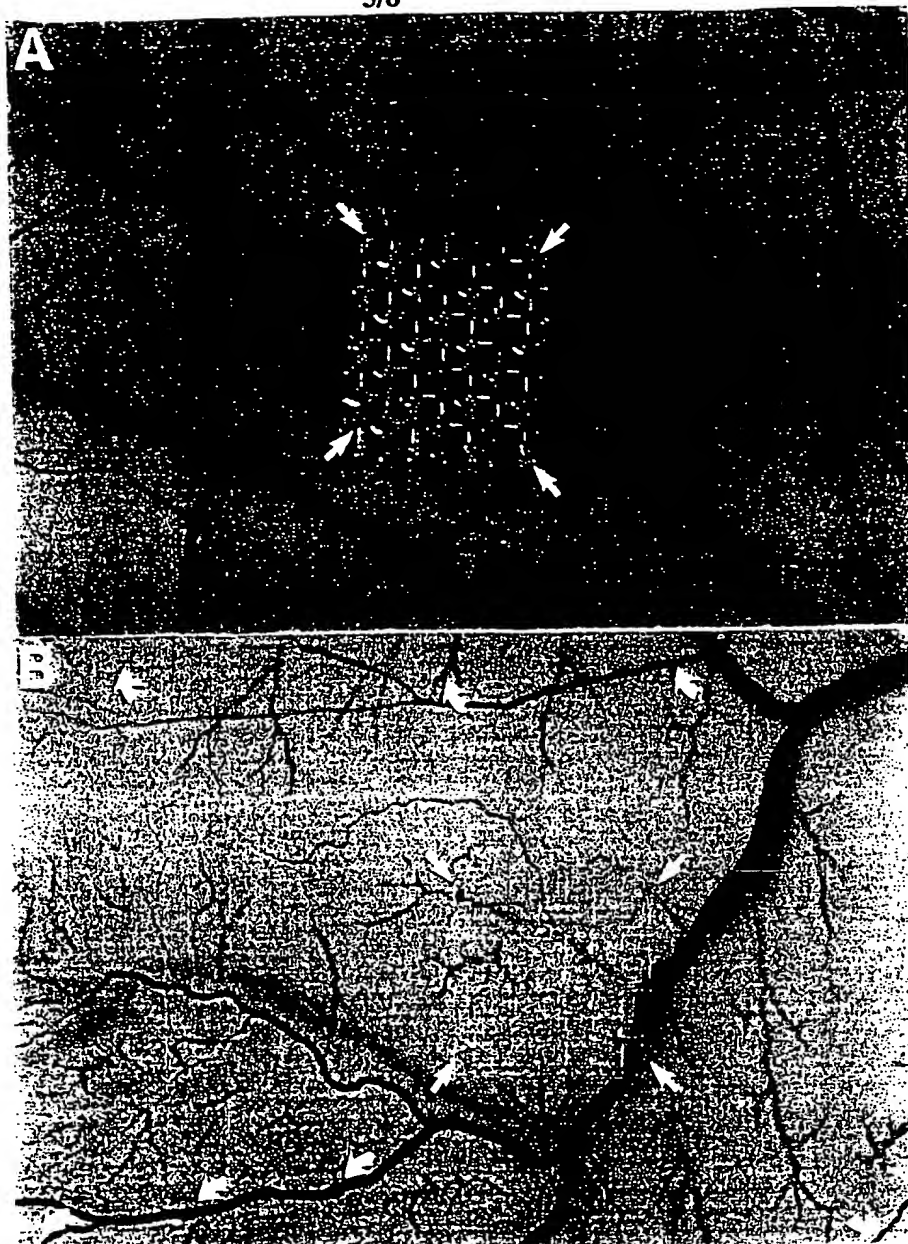
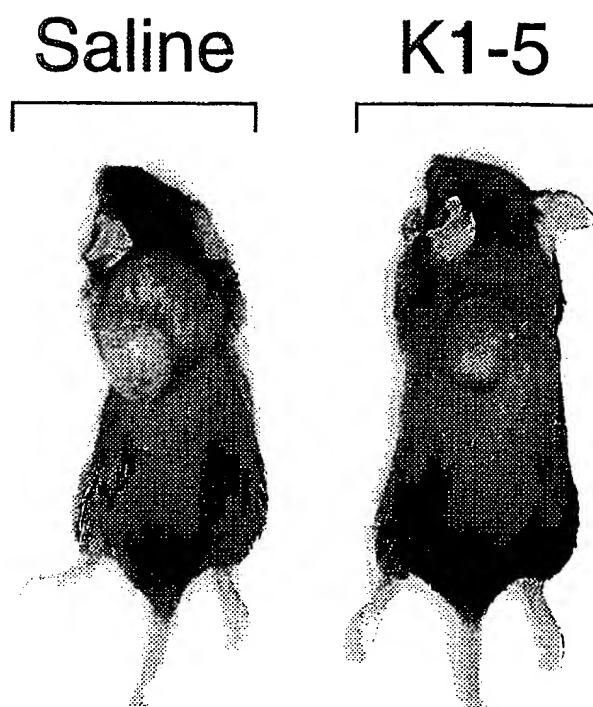


Fig. 6A



7/8

Fig. 6B

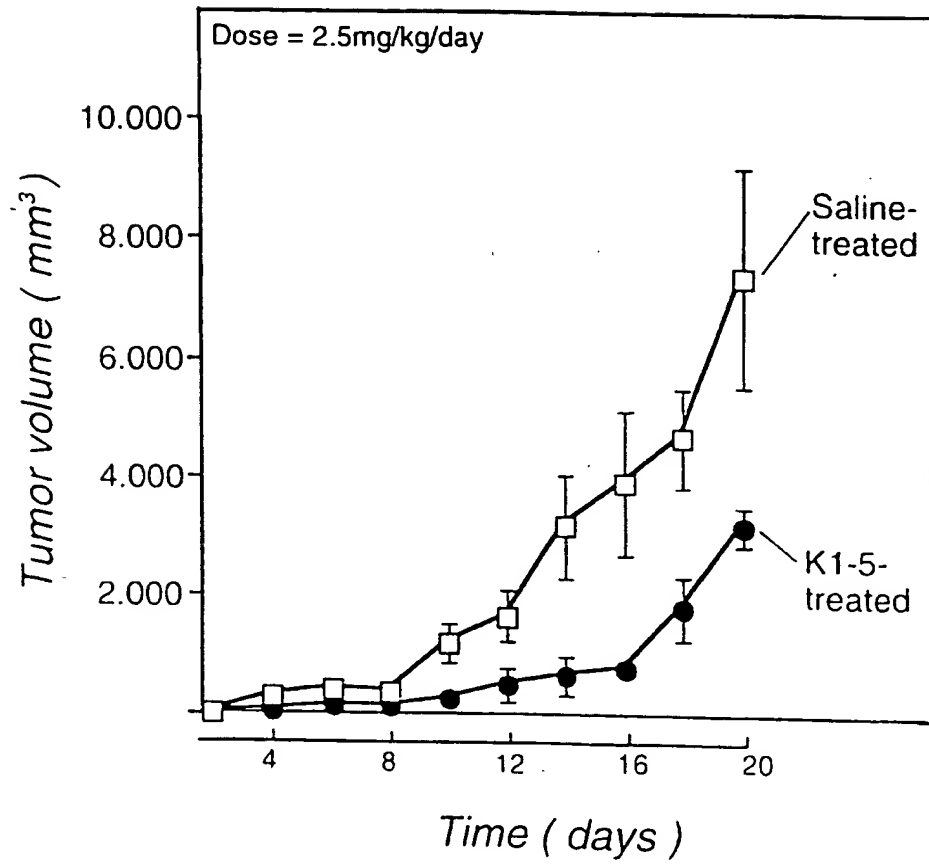
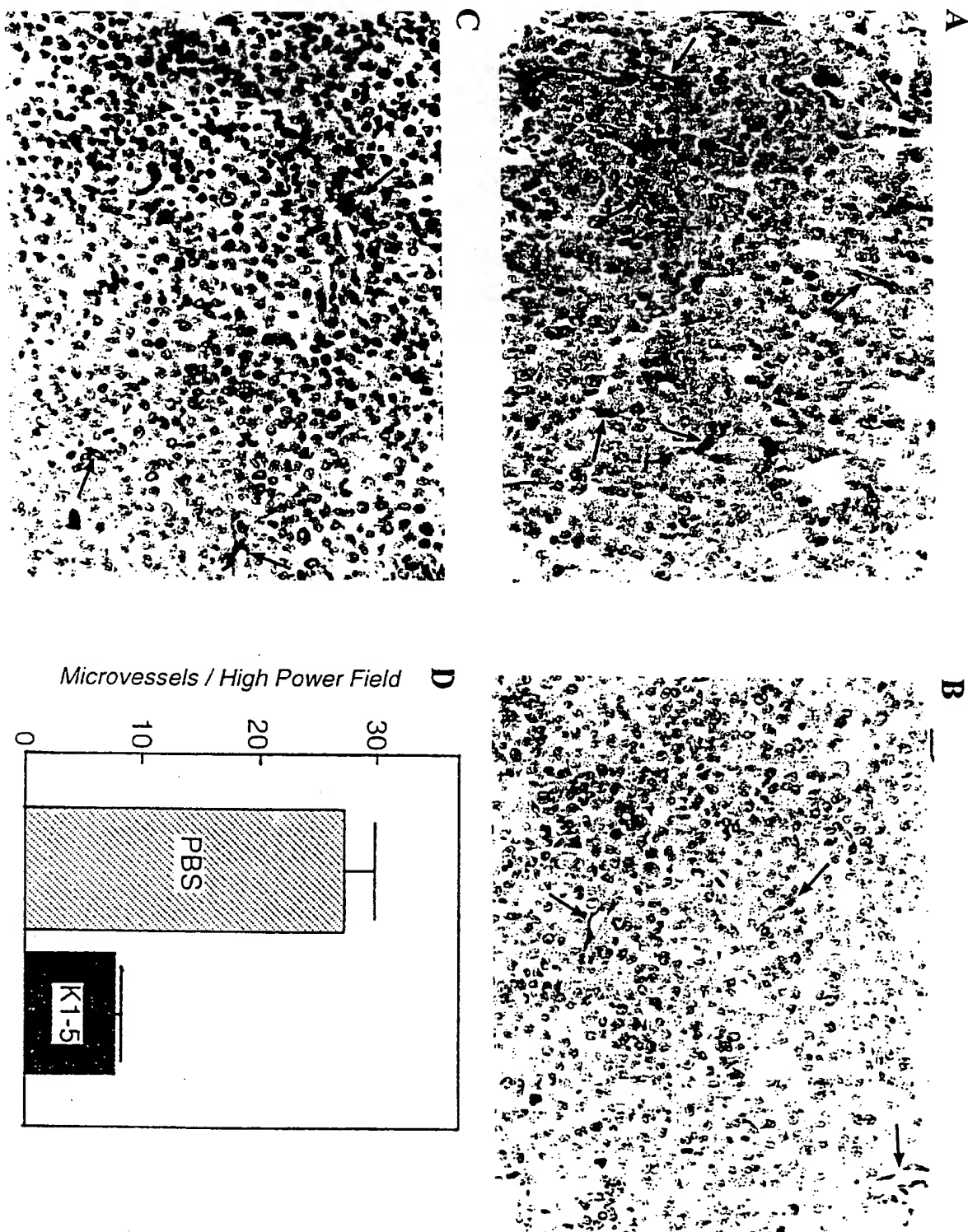


Fig. 7





## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01262

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/47, A61K 38/17

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CANCERLIT, WPI, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9815574 A1 (NORTHWESTERN UNIVERSITY), 16 April 1998 (16.04.98) --	1-16
P,X	WO 9741824 A2 (ABBOTT LABORATORIES), 13 November 1997 (13.11.97) --	1-16
X	The Journal of Biological Chemistry, Volume 265, No 32, 1990, Hua-Lin Wu et al, "Interaction of Plasminogen and Fibrin in Plasminogen Activation", page 19658 - page 19664 --	1-6,12
P,A	WO 9723500 A1 (THE CHILDREN'S MEDICAL CENTER CORPORATION), 3 July 1997 (03.07.97) --	1-16

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

28 Sept 1998

Date of mailing of the international search report

29-09-1998

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Hampus Rystedt

Telephone No. +46 8 782 25 00

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

27/07/98

International application No.

PCT/SE 98/01262

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9815574 A1	16/04/98	AU 4422797 A	05/05/98
WO 9741824 A2	13/11/97	AU 3060697 A	26/11/97
WO 9723500 A1	03/07/97	AU 1687097 A	17/07/97